

**CERTIFICATE**

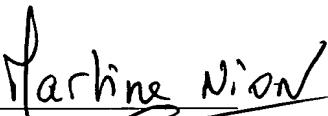
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do hereby declare that I am conversant with the French and English Languages, and that the attached translation signed by me is, to the best of my knowledge and belief, a true and correct translation of International Patent Application No. PCT/FR 03/00157 filed on January 17, 2003.

Dated : June 14, 2004

Signed :

  
Martine NION

METHOD FOR IDENTIFYING SUBSTANCES CAPABLE OF MODULATING  
ADIPOCYTE DIFFERENTIATION

The present invention concerns methods for screening active molecules, in particular  
5 molecules having an activity in modulating adipocyte differentiation. The invention also  
concerns genetic constructs, cells and compositions useful for carrying out such screening  
methods, for example genetically modified pre-adipocyte cells overexpressing the *REV-*  
*ERB ALPHA* receptor, and methods for preparing said cells. The invention is useful for  
identifying active compounds or compounds that can serve as leads for developing active  
10 medicaments for managing metabolic disorders, in particular for treating diabetes,  
obesity, insulin resistance and/or syndrome X.

In particular, the invention is based on the demonstration and characterization of the role  
of a specific nuclear receptor, *REV-ERB ALPHA*, in the mechanisms of adipocyte  
15 differentiation, and particularly on the ability of said receptor, when it is overexpressed,  
to sensitize cells to the action of adipocyte differentiation factors. The invention is also  
based on the production of specific vectors allowing expression of the *REV-ERB ALPHA*  
receptor, and on genetically modified cell lines, particularly pre-adipocytes. The results  
obtained reveal a modulation of adipocyte differentiation of such lines when they are  
20 contacted with agonists or antagonists of receptors directly or indirectly involved in the  
adipocyte differentiation process.

White adipose tissue is the principal site of energy storage in eukaryotes. Its role is to  
store triglycerides during periods of abundance and mobilize them when energy supplies  
25 diminish. A deregulation of adipocyte activity leads to obesity and its consequences such  
as non-insulin-dependent diabetes. The adipocytes constituting white adipose tissue are  
highly specialized cells expressing a defined set of genes characteristic of their  
differentiation (Fajas et al., Curr. Opin. Cell Biol. 1998, 10: 165-173; Spiegelman,  
Diabetes 1998, 47: 507-514; Gregoire F., Phys. Rev. 1998, 78: 783-809).

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Adipocyte differentiation is a complex process the molecular components of which are  
becoming more clearly understood (Fajas et al., Curr. Opin. Cell Biol. 1998, 10: 165-173;  
Spiegelman, Diabetes 1998, 47: 507-514; Gregoire F., Phys. Rev. 1998, 78: 783-809).

Adipocyte differentiation is subjected to a coordinated regulation by a network of several transcription factors. It is initiated by exit from the cell cycle and activation of the factors C/EBP beta, C/EBP delta and ADD1 (SREBP1c), which induce expression of the nuclear receptor activated by "peroxisome proliferators" of the gamma type, hereinbelow named 5 PPAR GAMMA, the principal coordinator of adipocyte differentiation.

The PPAR GAMMA receptor stimulates exit from the cell cycle and expression of genes specific of adipocytes which allow storage of energy. Finally, the C/EBP alpha transcription factor cooperates with the PPAR GAMMA receptor in the final steps of 10 adipocyte differentiation to induce a new set of genes and to maintain expression of said PPAR GAMMA receptor.

The *REV-ERB ALPHA* receptor is an orphan nuclear receptor the natural or artificial ligands of which are unknown. Its sequence is encoded on the non-coding strand of the 15 gene coding for the thyroid hormone receptor type alpha (Lazar, M.A. et al., Mol. Cell. Biol. 1989, 9(3): 1128-1136; Lazar, M.A. et al., DNA Cell Biol. 1990, 9(2): 77-83; Laudet, V. et al., Nucleic Acid Res. 1991, 19(5): 1105-1012). It acts mainly as a transcription inhibitor. Its expression appears to increase during differentiation of pre-adipocytes to adipocytes and is correlated with the expression of markers of adipocyte 20 differentiation (Chawla, J. Biol. Chem. 1993, 266,12: 16265-16269).

The *REV-ERB ALPHA* receptor acts as a negative transcriptional regulator (Laudet, V. et al., Curr. Biol. 1995, 5(2): 124-127). It has been shown that the human *REV-ERB ALPHA* receptor regulates its own expression (Adelman, G. et al., Proc. Natl. Acad. Sci. USA 25 1996, 93(8): 3553-3558). *REV-ERB ALPHA* receptor mRNA is highly expressed in tissues such as adipose tissue, striated muscle, liver and brain tissue and less abundantly in other tissues.

The *REV-ERB ALPHA* receptor is induced during adipocyte differentiation (Chawla, A. et 30 al., J. Biol. Chem. 1993, 268(22): 16265-16269). However, the molecular mechanism of this regulation remains obscure. It has also been observed that the *REV-ERB ALPHA* receptor is involved in muscle differentiation (Downes M. et al., Mol. Endocrinol. 1995, 9(12): 1666-1678) and in the regulatory mechanism of lipid metabolism, due to the

identification of the rat apoA1 gene (gene encoding apolipoprotein A1) as target gene of the *REV-ERB ALPHA* receptor in liver (Vu-Dac, N., J. Biol. Chem. 1998, 273: 25713-25720). It has further been suggested that the *REV-ERB ALPHA* receptor acts as a modulator of thyroid hormone signaling (Lazar M.A., J. Biol. Chem. 1990, 265(22): 5 12859-12863; Munroe, S.H. et al., J. Biol. Chem. 1991, 266(33): 22803-22086). In fact, the *REV-ERB ALPHA* receptor binds to the DR4 hormone response element (Spanjaard, R.A. et al., Mol. Endocrinol. 1994, 8(3): 286-295) and inhibits formation of the TR homodimer and TR/RXR heterodimers of TRES (Downes, M. et al., Mol. Endocrinol. 1995, 9: 1666-1678).

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So far, the biological role of the *REV-ERB ALPHA* receptor in adipose tissue and its mechanism of action have not yet been clarified (Chawla, A. et al., J. Biol. Chem. 1993, 268(22): 16265-16269).

15

The work carried out by the inventors has now elucidated interactions between two receptors, *REV-ERB ALPHA* and PPAR GAMMA. These studies showed that the PPAR GAMMA receptor activates transcription of the Rev-erb alpha gene via the DR2 response element of the Rev-erb alpha gene promoter (named "Rev-DR2"). The inventors were therefore able to determine that the Rev-erb alpha gene is a target of the PPAR GAMMA 20 receptor, that the *REV-ERB ALPHA* receptor is a promoter of adipocyte differentiation induced by the PPAR GAMMA receptor, and that it plays a modulating role in adipogenesis.

20

The inventors have also shown that, in a surprising manner, overexpression of the *REV-ERB ALPHA* receptor in pre-adipocytes, such as the 3T3-L1 cell line, increases the differentiation of said pre-adipocytes and increases the expression of the PPAR GAMMA receptor in said cells.

30

In this manner, the inventors have identified regulatory mechanisms between *REV-ERB ALPHA* receptors and other receptors involved in the adipocyte differentiation program, particularly the PPAR GAMMA receptor. On the basis of this work, they now propose a novel method for screening compounds capable of interacting either with the *REV-ERB*

*ALPHA* receptor or with said other receptors involved in the adipocyte differentiation program.

Said method is useful for identifying active compounds for treating pathologies linked to 5 metabolic abnormalities involving said receptors, such as adipocyte differentiation, diabetes, obesity, insulin resistance and syndrome X.

It is known that some compounds used for treating disorders linked to abnormalities of 10 adipocyte differentiation, such as diabetes or obesity, exert their action by interacting with the PPAR GAMMA receptor. For example, the thiazolidinediones, also known as glitazones – used to treat insulin resistance – have been identified as ligands and artificial activators of the PPAR GAMMA receptor. Also, fatty acid derivatives have been identified as natural ligands of the PPAR GAMMA receptor. Fibrates are also potent regulators of lipid metabolism which act as activators of the PPAR ALPHA receptor.

15 The results of *in vivo* and *in vitro* studies carried out by the inventors and described herein show that treatment with rosiglitazone (also named BRL49653 or BRL) increases the expression of the mRNA coding for the *REV-ERB ALPHA* receptor. The glitazones, antidiabetic agents frequently used in the treatment of type 2 diabetes, therefore induce 20 the adipocyte differentiation program via binding and activation of the PPAR GAMMA nuclear receptor.

25 A first aspect of the invention therefore concerns methods for screening active molecules, in particular molecules having an activity in modulating adipocyte differentiation, based on the use of the *REV-ERB ALPHA* receptor as molecular target.

30 Another aspect of the invention relates to genetic constructs, cells and compositions useful for practicing said screening methods, for example pre-adipocyte cells genetically modified to overexpress the *REV-ERB ALPHA* receptor, and methods for preparing said cells.

A specific aspect of the invention also concerns recombinant viruses (or viral vectors) coding a *REV-ERB ALPHA* polypeptide.

A further aspect of the invention concerns the use of active compounds for carrying out methods of therapeutic or vaccinal treatment of the human or animal body. In particular, these are compounds capable of interfering with the binding of the PPAR GAMMA receptor to the Rev-DR2 site or, more generally, with the activity or expression of the *REV-ERB ALPHA* receptor in adipocyte differentiation.

**REV-ERB ALPHA receptor**

10 The invention is based in particular on the identification of the role of the *REV-ERB ALPHA* receptor in adipocyte differentiation, on the characterization of the mechanisms underlying said role, and on the use of this molecule for a therapeutic purpose.

15 In the spirit of the invention, the term *REV-ERB ALPHA* receptor denotes a nuclear receptor comprising the primary amino acid sequence SEQ ID NO : 4, or a fragment or functional variant thereof.

20 MTTLDNNNTGGVITYIGSSGSSPSRTSPESLYSDNSNGSFQSLTQGCPTYFPPSPTGSLTQDP  
 ARSGSIPPSLSDGSPSSSSSSSSSSSFYNGSPPGSLQVAMEDSSRVSPSKSTSNTKLNGMV  
 LLCKVCGDVASGFHYGVHACEGCKGFFRRSIQQNIQYKRCLKNENCSIVRINRNRCQQCRFKKC  
 LSVGMSRDAVRFGRIPKREKQRLAEMQSAMNLANNQLSSQCPLETSPTQHPTPGPMGPSPPPP  
 APVPSPLVGFSQLPQLTPPRSPSPEPTVEDVISQVARAHREIFTYAHDKLGSSPGNFNANHASG  
 SPPATTPHRWENQGCPPAPNDNNTLAAQRHNEALNGLRQAPSSYPPTWPPGPAHHSCHQNS  
 NGHRLCPHTVYAAPEGKAPANSPRQGNSKNVLLACPMNMYPHGRSGRTVQEIWEDFSMSFTP  
 25 AVREVVEFAKHIPGFRDLSQHDQVTLLKAGTFEVLMVRFAFLNVKDQTVMFSLRTTYSLQELG  
 AMGMGDLLSAMFDSEKLNSLALTEEEGLFTAVVLSADRSGMENSASVEQLQETLLRALRAL  
 VLKNRPLETSRFTKLLLKLPLRTLNNMHSEKLLSFRVDAQ (séquence SEQ ID NO :4)

30 The term “fragment” typically designates a polypeptide comprising from 5 to 200 consecutive amino acids of SEQ ID NO : 4, preferably from 5 to 150, even more preferably from 5 to 100. Particular examples of fragments are polypeptides of 5 to 80 amino acids. Preferably, the fragments comprise a functional domain of sequence SEQ ID NO : 4, for example a transcription inhibitor domain and/or a DNA binding domain. The term “functional variant” encompasses natural variants, particularly those resulting 35 from polymorphism(s), splicing(s), interspecies variation(s), and the like. Said term also includes synthetic variants, particularly polypeptides comprising a sequence derived from sequence SEQ ID NO : 4 by one or more mutations, deletions, substitutions and/or

additions of one or more residues. In a preferred manner, a synthetic variant shows 75 % primary sequence homology with sequence SEQ ID NO : 4, even more preferably, at least 85 %. The fragments or variants may further contain added heterologous regions or chemical, enzymatic, immunologic, modifications, etc. For instance, said modifications 5 may facilitate the production or purification of the receptor, improve its stability, increase its activity, etc.

In a preferred embodiment of the invention, the term *REV-ERB ALPHA* receptor designates a receptor of human origin, particularly a receptor comprising sequence SEQ 10 ID NO : 4 or a fragment thereof.

The term “Rev-erb alpha gene” generally denotes any portion of the genome coding a *REV-ERB ALPHA* receptor such as defined hereinabove.

15 The term “Rev-erb alpha genetic construct” or “recombinant nucleic acid coding a *REV-ERB ALPHA* receptor” generally designates any nucleic acid encoding a *REV-ERB ALPHA* receptor such as defined hereinabove. It may be a DNA or an RNA, for example a genomic DNA, cDNA, mRNA, synthetic or semi-synthetic DNA. These may be obtained by cloning from libraries or plasmids, or by synthesis, or by any other method 20 known to those skilled in the art.

In a particular embodiment of the invention, the Rev-erb alpha genetic construct is a nucleic acid comprising sequence SEQ ID NO : 3, a fragment thereof, or any sequence hybridizing with the above under conditions of moderate stringency and encoding a *REV-ERB ALPHA* receptor.

atg acgaccctgg actccaacaa caacacaggt  
 661 ggcgcatca cctacattgg ctccagtggc tcctcccaa gccgcaccag ccctgaatcc  
 721 ctctatagtg acaactccaa tggcagctc cagtcctga cccaggctg tcccacctac  
 781 ttccccacat ccccccactgg ctccctcacc caagaccgg ctcgctcctt tgggagcatt  
 841 ccacccaaagcc tgagtatgta cggctccct tcttcctcat cttccctgtc gtcatcctcc  
 901 tcctcccttataatggag ccccccggg agtctacaag tggccatgga ggacagcagc  
 961 cgagtgtccc ccagcaagag caccagcaac atcacaaggc tgaatggcat ggtgttactg  
 1021 tgtaaagtgt gtggggacgt tgccctgggc ttccactacg gtgtgcacgc ctgcgagggc  
 1081 tgcaagggtt tttccgtcg gagcatccag cagaacatcc agtacaaaag gtgtctgaag  
 1141 aatgagaatt gctccatcgt ccgcataat cgcaaccgct gccagcaatg tcgcttcaag

1201 aagtgtctct ctgtggccat gtctcgagac gctgtgcgtt ttgggcgcatt ccccaaacga  
 1261 gagaagcgc ggtatgctgc tgagatgcag agtgccatga acctggccaa caaccagttg  
 1321 agcagccagt gccccgttga gacttcaccc acccagcacc ccacccagg ccccatgggc  
 1381 ccctcgccac cccctgctcc ggtccctca cccctgggtgg gcttctccca gtttccacaa  
 5 1441 cagctgacgc ctcccagatc cccaaggccct gagcccacag tggaggatgt gatatccag  
 1501 gtggcccccggg cccatcgaga gatcttcacc tacgcccattt acaagctggg cagtcaccc  
 1561 ggcaacttca atgccaacca tgcatcaggt agccctccag ccaccacccc acatcgctgg  
 1621 gaaaatcagg gctgcccacc tgccccaat gacaacaaca ctttgcgtgc ccagcgtcat  
 1681 aacgaggccc taaatggctc ggcgcaggct ccctccttcc accctccac ctggccttcc  
 10 1741 ggccctgcac accacagctg ccaccaggctt aacagcaacg ggcaccgtct atgccccacc  
 1801 cacgtgtatg cagccccaga aggcaaggca cctgccaaca gtccccggca gggcaactca  
 1861 aagaatgttc tgctggcatg tcctatgaac atgtacccgc atggacgcag tggcgaacg  
 1921 gtgcaggaga tctgggagga ttctccatg agcttcacgc ccgctgtgcg ggaggtggta  
 1981 gagtttgc aacacatccc gggctccgt gaccttctc agcatgacca agtcaccc  
 15 2041 cttacggctg gcaccccttga ggtgctgtatg gtgcgtttt cttcggtt caacgtgaag  
 2101 gaccagacag ttagtgcattt aagccgcacc acctacagcc tgccaggatg tggcgcatt  
 2161 ggcattgggag acctgcttag tgccatgttc gacttcacgc agaagctaa cttccctggcg  
 2221 cttacccagg aggagctggg cttcttcacc ggggtggcgtc ttgtctgc agaccgctcg  
 2281 ggcattggaga attccgcctt ggtggagcag ctccaggaga cgctgtgcg ggcttccgg  
 2341 gctctggcgtc tgaagaaccc gcccctggag acttcccgct tcaccaagct gctgctcaag  
 2401 ctggccgacc tgcggaccctt gaacaacatg cattccgaga agctgtgtc cttccgggtg  
 2461 gacgcccagt ga (SEQ ID NO: 3)

Conditions of moderate stringency are described for example in Maniatis et al. The  
 25 following conditions are given as an example : incubation at 42°C for 12 hours in a  
 medium containing 50 % formamide, 5 x SSPE, 5 x Denhardt's solution, 0.1 % SDS.

Typically, the nucleic acid used for the recombination (recombinant nucleic acid) or  
 30 genetic construct comprises, in addition to a region coding the *REV-ERB ALPHA*  
 receptor, one or more transcriptional regulatory regions, typically a transcriptional  
 promoter and/or terminator. Said regulatory regions are selected according to the host  
 cell used. Preferably, they are regulatory regions that function in mammalian cells.  
 Examples include constitutive or regulated promoters, inducible or not, tissue-selective or  
 ubiquitous, strong or weak, such as for example viral promoters (for example : CMV,  
 35 LTR, SV40) or from cellular genes. In a particular embodiment, the promoter is the  
 promoter of the Rev-erb alpha gene, comprising for example sequence SEQ ID NO : 1 or  
 a region thereof, for instance a promoter comprising the sequence  
 AAAAGTGTGTCACTGGGGCA (SEQ ID NO : 2).

40 In a particular embodiment of the invention, the Rev-erb alpha genetic construct is a  
 nucleic acid comprising sequences SEQ ID NO : 3 and SEQ ID NO : 2, a fragment thereof

or any sequence hybridizing thereto under conditions of moderate stringency. In a more specific embodiment, the Rev-erb alpha genetic construct is a nucleic acid comprising a sequence coding a polypeptide SEQ ID NO : 4 operationally linked to a transcriptional promoter comprising sequence SEQ ID NO : 1 or a fragment thereof, particularly a transcriptional promoter comprising sequence SEQ ID NO : 2.

5 **Genetically modified cells**

10 A particular object of the present invention is based on a cell population comprising a recombinant nucleic acid coding a *REV-ERB ALPHA* receptor.

15 The cells may be any cell that can be cultured, preferably mammalian, for example human. They may be primary cells or established cell lines. Preferably, the host cells are pre-adipocyte cells. Said cells are generally defined as cells of the fibroblast type, which are capable of differentiating to adipocytes under suitable culture conditions. More specifically, they are mesodermal cells, incapable of differentiating to chondroblasts, osteoblasts or myoblasts and which, under favorable conditions specific to the cell in question, differentiate to adipocytes and express several differentiation markers characteristic of adipocytes. Examples of pre-adipocyte cells useful for practicing the 20 invention are in particular the cell lines 3T3-L1 (ATCC reference : CL-173), 3T3-F442A (Green H. et al., Cell 1975, 5:19-27), ob17 (Negrel R. et al., Proc. Natl. Acad. Sci. USA, 1978, 75: 6054-6058) or ob1771 (Doglio A. et al., Biochem J., 1986, 238: 123-129).

25 A particular object of the invention is therefore based on a genetically modified pre-adipocyte cell, wherein it comprises a recombinant nucleic acid coding a *REV-ERB ALPHA* receptor.

30 Other examples of cells that may be used within the scope of the invention are prokaryote cells, yeast cells or mammalian cells, particularly embryonic cells or cells such as CHO cells, fibroblasts, Vero cells, and the like.

The recombinant nucleic acid present in the cells enables said cells to express a *REV-ERB ALPHA* receptor, or to overexpress said receptor, when a basal expression is already

present in the cells. Thus, in the case of pre-adipocyte cells, the nucleic acid generally allows the cells to overexpress a *REV-ERB ALPHA* receptor, that is to say, to produce the receptor at a level higher than that observed in the same cells in the absence of the recombinant nucleic acid construct. The term overexpression generally denotes an expression increased by a factor of 2, more generally by a factor of 3, ideally by a factor of at least 5. The cells are preferably mammalian cells, in particular human cells. It is understood that cells from other species may be used, such as mouse, rat, monkey, hamster cells and the like, for example.

5

10 A particular object of the invention concerns a genetically modified cell, particularly pre-adipocyte, overexpressing the *REV-ERB ALPHA* receptor. The term genetically modified indicates that the cell (or an ancestor thereof) was modified to contain a recombinant nucleic acid coding said receptor.

15 Typically, the recombinant nucleic acid or genetic construct comprises, in addition to a region coding the *REV-ERB ALPHA* receptor, one or more transcriptional regulatory regions, typically a transcriptional promoter and/or terminator, such as defined hereinabove. The nucleic acid may be present or incorporated in a plasmid, viral vector, etc. It may be integrated in the cellular genome, or remain in extra-chromosomal form

20 (replicative or not).

The invention also has as its object a method for preparing recombinant cells expressing a *REV-ERB ALPHA* receptor, particularly genetically modified pre-adipocyte cells overexpressing a *REV-ERB ALPHA* receptor or a recombinant nucleic acid such as defined hereinabove. The inventive method generally comprises introducing a recombinant nucleic acid such as defined hereinabove coding a *REV-ERB ALPHA* receptor in a host cell. The host cells may be any cell population such as described hereinabove, preferably a pre-adipocyte, in particular the cell lines 3T3-L1, 3T3-F442A, ob17 or ob1771.

25

30 According to a first preferred embodiment of the invention, the recombinant cells are obtained by transfecting host cells with a plasmid vector comprising a Rev-erb alpha genetic construct. In an advantageous manner, the transfection is carried out in the

presence of a second genetic construct coding a selection or resistance gene, and the cells are selected for expression of said selection or resistance gene and of the nucleic acid coding REV ERB ALPHA.

5 In the spirit of the invention, the term “transfection” generally designates any method enabling the transfer of a nucleic acid into a cell. The method may be chemical, physical, biological, and the like. Examples include electroporation, calcium phosphate precipitation, use of agents that facilitate transfection, such as for instance lipids, polymers, peptides, etc., or else the use of physical techniques like “gene gun”, the use of  
10 projectile, bombardment, and the like.

In a particular embodiment, the method comprises cotransfected the cells with a plasmid vector comprising said recombinant nucleic acid and with a plasmid vector comprising an antibiotic resistance gene, the cells being selected for their resistance to said antibiotic  
15 and for their expression of said recombinant nucleic acid. According to a preferred embodiment of the invention, the recombinant cells are obtained by cotransfected host cells with a Rev-erb alpha genetic construct overexpressing the *REV-ERB ALPHA* receptor and with a genetic construct overexpressing an antibiotic resistance gene. The recombinant cells are then selected in the presence of the antibiotic and tested for  
20 overexpression of the *REV-ERB ALPHA* receptor.

According to a particular embodiment of the invention, the antibiotic used is selected in the group consisting of the following substances, which are given as non-limiting examples : neomycin, zeocin, hygromycin, blasticidin, etc.

25 In a particular embodiment of the method, the nucleic acid is introduced by transfection with a plasmid vector also comprising an antibiotic resistance gene, the cells being selected for their resistance to said antibiotic and for their expression of the recombinant nucleic acid.

According to this variant of the invention, the Rev-erb alpha genetic construct allowing  
30 overexpression of the *REV-ERB ALPHA* receptor also comprises a functional cassette which allows overexpression of an antibiotic resistance gene. The recombinant cells are then selected in the presence of the antibiotic and tested for their overexpression of the *REV-ERB ALPHA* receptor. In a particular embodiment of the invention, the antibiotic

used is selected in the group consisting of the following substances, which are given as non-limiting examples : neomycin, zeocin, hygromycin, blasticidin, etc.

In another embodiment, the nucleic acid is introduced by transfecting with a plasmid  
5 vector which additionally comprises an antibiotic resistance gene and a eukaryotic origin  
of replication, the cells being selected for their resistance to said antibiotic and for their  
expression of the recombinant nucleic acid. In a more specific embodiment, the Rev-erb  
alpha genetic construct allowing overexpression of the *REV-ERB ALPHA* receptor  
therefore also comprises a functional cassette allowing overexpression of an antibiotic  
10 resistance gene and a eukaryotic origin of replication. The recombinant cells are then  
selected in the presence of the antibiotic and tested for overexpression of the *REV-ERB*  
*ALPHA* receptor. According to a particular embodiment of the invention, the antibiotic  
used is selected in the group consisting of the following substances, which are given as  
non-limiting examples : neomycin, zeocin, hygromycin, blasticidin, etc.

15

According to another preferred embodiment of the invention, the nucleic acid is introduced into the cells by infection with a viral vector comprising said nucleic acid.

20 According to an especially preferred embodiment of the invention, the introduction is accomplished by using a recombinant virus comprising the recombinant nucleic acid coding the *REV-ERB ALPHA* receptor and, as the case may be, the selection or resistance gene ("infection").

Different types of recombinant virus may be used, such as for example retroviruses,  
25 adenoviruses, AAV (Adenovirus Associated Virus), herpes viruses, modified baculoviruses, etc. Preferred recombinant viruses are recombinant adenoviruses and retroviruses.

30 In a preferred embodiment, the recombinant cells (advantageously overexpressing the RNA coding for the *REV-ERB ALPHA* receptor) are obtained by infecting the host cells, particularly pre-adipocytes, with viral vectors, preferably adenovirus or retrovirus, said vectors containing a nucleic acid coding a *REV-ERB ALPHA* receptor.

In this respect, another object of the invention is a viral vector comprising a nucleic acid coding a *REV-ERB ALPHA* receptor. Another object of the invention is a recombinant virus comprising, in its genome, a nucleic acid coding a *REV-ERB ALPHA* receptor. Preferably, the viral vector is a replication-defective vector, that is to say, incapable of 5 autonomously replicating in a cell. Typically, a viral vector is defective for one or several viral genes essential for replication. In the case of retroviruses, the main viral genes are the gag, pol and env genes. In the case of adenoviruses, the principal genes are contained in the E1A, E1B, E4 and E2 regions. In AAV, the Rep and Cap genomic regions are concerned. The construction of viral vectors, defective for one or several (or 10 all) viral genes and comprising a nucleic acid of interest is known to those skilled in the art. For instance, said methods make use of packaging cell lines and/or vectors or helper virus, as illustrated in the examples.

A particular object of the invention concerns :

15 - a defective recombinant adenovirus comprising, in its genome, a nucleic acid coding a *REV-ERB ALPHA* receptor. The adenovirus is preferably a group C adenovirus, particularly Ad5, and/or advantageously comprises a deletion of all or part of the E1A and/or E1B and/or E4 region;

20 - a defective recombinant retrovirus comprising, in its genome, a nucleic acid coding a *REV-ERB ALPHA* receptor. The retrovirus is preferably a retrovirus derived from MLV (Mouse Leukemia Virus) or a lentivirus, and/or advantageously bears a deletion of all or part of the gag and/or pol and/or env region.

25

According to a particular embodiment of the invention, the preparation (e.g., transfection, infection) of cells, particularly of pre-adipocytes, is carried out with a Rev-erb alpha genetic construct which contains, in addition to a region coding the *REV-ERB ALPHA* receptor, for example SEQ ID NO : 3, one or more transcriptional regulatory regions, 30 typically a transcriptional promoter and/or terminator. According to a preferred embodiment of the invention, these are regulatory regions functional in mammalian cells. Non-limiting examples include constitutive or regulated promoters, inducible or not,

tissue-selective or ubiquitous, strong or weak, such as for instance viral promoters (for example : CMV, LTR, SV40) or from cellular genes.

According to a particular embodiment of the invention, the preparation (e.g., transfection) 5 of cells (e.g., pre-adipocytes) is carried out with a Rev-erb alpha genetic construct which comprises, in addition to a region coding the *REV-ERB ALPHA* receptor, for example SEQ ID NO : 3, the promoter of the Rev-erb alpha gene, for example comprising sequence SEQ ID NO : 1, or comprising a region thereof, for example SEQ ID NO : 2. In another particular embodiment, the preparation is accomplished with a sequence selected 10 from or comprising sequences SEQ ID NO : 1 and SEQ ID NO : 3.

To prepare the recombinant cells of the invention, the host cells may be contacted with the Rev-erb alpha gene or recombinant nucleic acid or vector or virus in any suitable condition, after which the recombinant cells are recovered. Contact may be carried out on 15 any suitable support and in any culture medium suitable to the cell type (for example : DMEM, RPMI, etc.).

In a particular embodiment, after infection or transfection, stable cultured cell lines are selected. The preferred genetically modified cells overexpressing the gene coding the 20 *REV-ERB ALPHA* receptor are stable lines.

#### Screening methods

The present invention also has as object methods for identifying, selecting, characterizing 25 or optimizing compounds capable of modulating adipocyte differentiation. Said methods may be carried out in cellular tests or *in vitro*, for example by binding tests. Said methods principally use a REV ERB ALPHA receptor (or a corresponding nucleic acid) as molecular target.

30 In a first embodiment, the invention has as its object a method for identifying, selecting, characterizing or optimizing compounds capable of modulating adipocyte differentiation, wherein (i) a compound to be tested is contacted with cells (preferably pre-adipocytes) such as defined as hereinabove, (ii) adipocyte differentiation of said cells is measured or

determined and (iii) preferably, said differentiation is compared with adipocyte differentiation of the same said cells in the absence of said compound to be tested.

The cells are preferably pre-adipocyte cells such as defined hereinabove, more

5 particularly pre-adipocyte cells (over)-expressing the *REV-ERB ALPHA* receptor.

According to a preferred embodiment of the inventive method, the compound to be tested is contacted with cells (preferably genetically modified cells overexpressing the *REV-ERB ALPHA* receptor) in the presence or absence of at least one activator of a receptor  
10 involved in the adipocyte differentiation program or at least one activator of a gene coding a receptor involved in the adipocyte differentiation program.

In fact, the results presented in the examples show in a surprising manner, that expression of the *REV-ERB ALPHA* receptor in the recombinant cells of the invention sensitizes pre-

15 adipocytes to the action of adipocyte differentiation factors and promotes the differentiation program. Under these conditions, selection of compounds modulating said differentiation is greatly facilitated.

In a first variant of the invention, one uses at least one activator of a receptor involved in

20 the adipocyte differentiation program, such as for example, and not by way of limitation, an activator of the PPAR GAMMA receptor. For example, the PPAR GAMMA receptor activator is selected in the group consisting of but not limited by : thiazolidinediones (rosiglitazone, troglitazone, englitazone, ciglitazone, pioglitazone, KRP-297), N-(2-benzoylphenyl)-L-tyrosines, 15-deoxy-delta-12,14-prostaglandin J2, etc.

25

In another variant, one uses at least one activator of a gene of a receptor involved in the adipocyte differentiation program. A non-limiting example is to contact the compound to

be tested with genetically modified cells overexpressing the *REV-ERB ALPHA* receptor in the presence or absence of at least one activator of the PPAR gamma gene. Preferably,

30 the activator of the PPAR gamma gene is selected in the group consisting of : C/EBP beta, C/EBP delta, ADD1 (SREBP1c).

The compound and the activator may be contacted with the cells at the same time, or successively. Typically, the activator is added first, followed by the test compound.

Adipocyte differentiation may be measured by staining the differentiated cells. For

5 example, the stain is selected in the group comprising the stains Oil Red O, Sudan Black.

Adipocyte differentiation may also be measured by determining the transport or synthesis of fatty acids.

Adipocyte differentiation may also be measured by determining the expression of at least one marker specific of differentiated adipocytes, preferably a marker selected in the group

10 consisting of : aP2, adiponectin and leptin.

The inventive method is noteworthy in that it makes it possible to :

- identify compounds capable of modulating the activity of the *REV-ERB ALPHA* receptor, such as compounds capable of modulating the expression of

15 the Rev-erb alpha gene or compounds that are agonists or antagonists of the *REV-ERB ALPHA* receptor. Thus, in particular the inventive method allows identification of compounds capable of increasing adipocyte differentiation and representing activators of Rev-erb alpha gene expression or agonists of the *REV-ERB ALPHA* receptor.

20 - indirectly identify, in the absence of PPAR gamma gene activator and PPAR GAMMA receptor activator, compounds capable of increasing adipocyte differentiation which act as PPAR GAMMA receptor agonists.

According to specific embodiments, the inventive method makes it possible to :

25 - identify compounds capable of decreasing adipocyte differentiation and representing *REV-ERB ALPHA* receptor antagonists.

- identify compounds capable of increasing adipocyte differentiation representing *REV-ERB ALPHA* receptor agonists

- identify, in the presence of PPAR gamma gene activator and/or PPAR GAMMA receptor activator, compounds capable of reducing adipocyte differentiation.

- identify compounds which are PPAR GAMMA receptor agonists.

It is understood that a receptor agonist or antagonist is a compound which binds to said receptor and respectively activates or inhibits the activity thereof.

The test compound may be of diverse origin and nature. It may be an isolated compound,  
5 biological extracts, organic or inorganic molecules, molecular libraries (synthetic, peptides, nucleic acids, etc.) or microorganisms, etc. The test compound may be contacted with the nucleic acid construct or cells on (or in) any suitable support and in particular on a plate, in a tube or flask, membrane, etc. In general, contact is carried out in a multiwell plate which allows numerous and various tests to be conducted in parallel.

10 Typical supports include microtiter plates and more particularly plates containing 96 or 384 wells (or more). Depending on the support and the nature of the test compound, variable quantities of cells may be used to practice the herein-described methods. Classically,  $10^3$  to  $10^6$  cells are contacted with a type of test compound, in a suitable culture medium, and preferably between  $10^4$  and  $10^5$  cells. The quantity (or  
15 concentration) of test compound may be adjusted by the user according to the type of compound (its toxicity, its ability to penetrate inside cells, etc.), the number of cells, the incubation time, etc. Generally, the cells are exposed to quantities of test compounds ranging from 1 nM to 1 mM. Of course it is possible to test other concentrations without deviating from the invention. Each compound may furthermore be tested at different  
20 concentrations, in parallel. Also, different adjuvants and/or vectors and/or agents that facilitate penetration of the compounds into cells may also be used, where necessary. Contact may be maintained for example for several minutes to several hours or days, in particular between 5 and 72 hours, generally between 12 and 48 hours.

25 According to another embodiment, the inventive method comprises selecting compounds capable of modulating expression of the *REV-ERB ALPHA* receptor, in particular modulating the effect of the PPAR GAMMA receptor on the Rev-erb alpha gene promoter. In fact, the inventors have now shown that the PPAR GAMMA receptor is responsible for modulating *REV-ERB ALPHA* receptor expression, and that said modulation involves an interaction between the PPAR GAMMA receptor and the Rev-erb  
30 alpha gene promoter, particularly at the Rev-DR2 region (SEQ ID NO : 2).

The invention also concerns a method for identifying, selecting, optimizing or characterizing compounds capable of modulating adipocyte differentiation, wherein it comprises (i) contacting a test compound and a nucleic acid comprising the Rev-DR2 sequence or a functional equivalent thereof, in the presence of the PPAR GAMMA receptor, (ii) verifying binding of the PPAR GAMMA receptor to said nucleic acid and, optionally, (iii) comparing said binding with that observed in the absence of test compound, the test compounds modulating PPAR GAMMA receptor binding being compounds modulating adipocyte differentiation.

10 Measurement of the eventual binding of the test compound, PPAR GAMMA receptor or a complex formed from the PPAR GAMMA receptor and said test compound to the response element may be carried out by any method known to those skilled in the art, for instance by detecting a signal produced by the response element after said binding. These may be direct or indirect methods, such as those using a reporter gene, binding tests, etc.

15 Thus, a particular method of the invention comprises contacting a test compound and a nucleic acid comprising the sequence Rev-DR2 (sequence SEQ ID NO : 1 or preferably SEQ ID NO : 2) or a functional equivalent thereof, in the presence of the PPAR GAMMA receptor, and verifying a binding of the PPAR GAMMA receptor to said nucleic acid. In 20 an advantageous manner the binding is compared with that observed in the absence of test compound. In another embodiment, the test compound and the PPAR GAMMA receptor are contacted with a reporter system comprising (i) a transcriptional promoter comprising one or more copies of sequence SEQ ID NO : 1, preferably of sequence SEQ ID NO : 2 or a functional variant thereof and (ii) a reporter gene, and the activity of the test 25 compound is determined by measuring its effect on reporter gene expression induced by the PPAR GAMMA receptor.

30 A particular object of the invention thus concerns a method for identifying, selecting, optimizing or characterizing compounds capable of modulating adipocyte differentiation, wherein it comprises contacting a test compound and the PPAR GAMMA receptor with a reporter system comprising (i) a transcriptional promoter comprising one or more copies of sequence SEQ ID NO : 1, preferably of sequence SEQ ID NO : 2 or a functional variant thereof and (ii) a reporter gene, and evaluating the activity of the test compound

by measuring its effect on reporter gene expression induced by the PPAR GAMMA receptor.

The reporter gene may be placed under the control of any promoter (for example, SEQ ID NO : 1) the sequence of which comprises sequence SEQ ID NO : 2 or a functional variant thereof. Said specific sequence may be present in one or more copies in the promoter (preferably 1 to 10 and even more preferably 1 to 6), upstream or downstream or internally, in the same orientation or in the opposite orientation. In a preferred embodiment of the invention, the reporter gene is placed under the control of a promoter comprising one or more copies of sequence SEQ ID NO : 2. Preferably, it is a promoter whose different activity in the absence and presence of the PPAR GAMMA receptor or a functional equivalent can be detected.

In this respect, the response element of the PPAR GAMMA receptor may be associated with a transcriptional minimal promoter. The minimal promoter is a transcriptional promoter having weak or nonexistent basal activity, and which can be increased in the presence of a transcriptional activator (for example the PPAR GAMMA receptor). A minimal promoter may therefore be a naturally weak promoter in mammalian cells, that is to say, producing a non-toxic and/or insufficient expression to obtain a noticeable biological effect. In an advantageous manner, a minimal promoter is a construct prepared from a native promoter, by deleting region(s) not essential to transcriptional activity. For instance, it is preferably a promoter comprising principally a TATA box, generally less than 160 nucleotides in size, centered around the transcription initiation codon. A minimal promoter may thus be prepared from strong or weak, viral, cellular promoters, such as for instance the herpes virus thymidine kinase (TK) gene promoter, the CMV immediate early promoter, the PGK promoter, the SV40 promoter, and the like.

In a preferred embodiment, the reporter gene is placed under the control of the Rev-erb alpha gene promoter, for example a promoter comprising the non-coding sequence of SEQ ID NO : 1.

Any reporter gene may be used in the inventive screening method. Among such, examples include the chloramphenicol acetyltransferase (CAT) gene, luciferase gene of

firefly (Luc) or Renilla (Ren), the secreted alkaline phosphatase (SAP) gene or that of beta-galactosidase ( $\beta$ -Gal). The activity of the proteins encoded by said genes can be readily measured by conventional methods and provides an indirect knowledge of the effect of nuclear receptors on gene expression by measuring the quantity of protein 5 produced and/or their enzymatic activity. The reporter system is advantageously introduced in a cell population, which may be prokaryotic or eukaryotic.

Another object of the invention is the use of a compound identified, selected, characterized or optimized according to a method described hereinabove for preparing a 10 medicament for carrying out a method of therapeutic or vaccinal treatment of the human or animal body, particularly a curative or preventive treatment of metabolic diseases, in particular diabetes, obesity, insulin resistance and syndrome X.

Another object of the invention is based on a method for preparing a medicament 15 comprising (i) a step of selecting a compound capable of modulating adipocyte differentiation such as described hereinabove and (ii) contacting a selected compound, or an analog thereof, with a pharmaceutically acceptable vehicle.

A further object of the invention is a method for preparing a compound active on 20 adipocyte differentiation, comprising (i) a step of selecting a compound capable of modulating adipocyte differentiation such as described hereinabove and (ii) synthesizing a selected compound, or an analog thereof.

25 Other aspects and advantages of the present invention will become apparent in the examples which follow and in the appended figures, which are given for purposes of illustration and not by way of limitation, in which :

- Figure 1 illustrates the results of Northern blot analyses of mRNA extracted from adipose tissue of rats treated or not with rosiglitazone (BRL) :
- 30 Adult male rats received either rosiglitazone (10 mg/kg/day) or excipient (1 % carboxymethylcellulose) for 14 days. Rats were sacrificed and dissected and total RNA was extracted from epididymal and perirenal adipose tissue. Ten micrograms

of mRNA were analyzed by Northern blot using *REV-ERB ALPHA* receptor cDNA (upper panel) or  $\beta$ -actin cDNA (lower panel) as probes.

5 - Figure 2 shows rosiglitazone (BRL) induction of *REV-ERB ALPHA* receptor mRNA expression in 3T3-L1 pre-adipocytes.

3T3-L1 pre-adipocytes were grown to confluence in DMEM medium supplemented with 10 % fetal calf serum. When the cells reached confluence, they were transferred to DMEM medium supplemented with 10 % fetal calf serum and stimulated with a mixture containing IBMX, dexamethasone, insulin, with or without rosiglitazone (1  $\mu$ M in water) for 9 days. RNA was isolated and analyzed by Northern blot.

10 - Figure 3 illustrates rosiglitazone (BRL) induction of activity of the Rev-erb alpha gene promoter and the PPAR GAMMA receptor.

15 3T3-L1 pre-adipocytes were transfected with a plasmid comprising a 1.7 kb fragment of the Rev-erb alpha gene promoter cloned in front of the luciferase reporter gene and with plasmid pSG5-PPAR gamma expressing the murine PPAR GAMMA receptor or with the corresponding empty pSG5 vector. Cells were treated with rosiglitazone (1  $\mu$ M) and luciferase activity was measured as previously described.

20

- Figure 4 illustrates the role of the Rev-DR2 site in induction of Rev-erb alpha gene promoter activity by the PPAR GAMMA receptor.

25 - Figure 4A shows the effects of the PPAR GAMMA receptor on the activity of a construct containing the promoter of the human *REV-ERB ALPHA* gene containing a wild type or mutant Rev-DR2 site.

- Figure 4B shows the effects of the PPAR GAMMA receptor on the activity of a construct containing the promoter of the human *REV-ERB ALPHA* gene containing a wild type or mutant Rev-DR2 site cloned in two copies upstream of the SV40 promoter.

30

Cos cells were transfected with the indicated reporter constructs and pSG5-PPAR-gamma or pSG5 plasmid. Cells were treated with rosiglitazone (BRL) and luciferase activity was measured.

- Figure 5 depicts the electrophoretic gel shift experiments on the PPAR gamma receptor which binds as a heterodimer with the RXR ALPHA nuclear receptor to the Rev-DR2 site of the Rev-erb alpha gene promoter.

The electrophoretic gel shift studies were carried out by using the indicated end-labeled 5 oligonucleotides, in the presence of the murine PPAR GAMMA receptor, murine RXR ALPHA receptor, human REV-ERB ALPHA receptor produced by reticulocyte lysate, or non-programmed lysates (lysate). Binding competition experiments were performed by adding a 0, 10 or 100-fold excess of unlabeled Rev-DR2 oligonucleotide.

10 - Figure 6 shows that exogenous expression of the *REV-ERB ALPHA* receptor stimulates lipid accumulation in 3T3-L1 cells.

3T3-L1 cells were infected with a control retrovirus (MFG-Neo) or with a retrovirus overexpressing the *REV-ERB ALPHA* receptor (MFG-Rev-erb alpha). The resulting cells 15 were induced to differentiate with or without 1  $\mu$ M rosiglitazone (BRL) for 8 days. Cells were then fixed and stained with Oil red O.

- Figures 6A, C and D show the microscopic views of the Oil red O-stained cells.
- Figure 6B shows macroscopic views of plaques stained with Oil red O.
- Figure 6E depicts the exogenous or endogenous expression of *REV-ERB ALPHA* 20 protein (Ecto-Rev or Endo-Rev) tested by Western blot.

A rabbit anti-*REV-ERB ALPHA* polyclonal antibody directed against a synthetic peptide (constituted by amino acids 263-365 of the human sequence) was used for the immunocytochemistry and Western blot experiments.

25 - Figure 7 illustrates the effect of exogenous expression of the *REV-ERB ALPHA* receptor on expression of the mRNA for the PPAR GAMMA receptor and the aP2 gene used as adipocyte differentiation marker. 3T3-L1 cells were infected with either MFG-Neo or MFG-REV retrovirus and treated for 8 days with a mixture containing IBMX and insulin (“Mix”), with or without 1  $\mu$ M rosiglitazone. mRNA was then extracted and analyzed by 30 Northern blot using the indicated probes.

Other advantages and features of the invention will become apparent in the following examples which describe the studies carried out by the inventors leading to the design and implementation of the screening method.

5      **1- MATERIALS AND METHODS**

**Materials**

Rosiglitazone (Ref. BRL49653) was provided by A. Bril (SKB, Rennes, France),

GP+E86 cells were from Columbia University (New York, NY, USA) and pMFG

10      plasmid from the Massachusetts Institute of Technology (Cambridge, MA, USA).

**Animals**

Ten-week-old male Sprague-Dawley rats received rosiglitazone (10 mg/kg/day) in

suspension in 1 % carboxymethylcellulose by gavage for 14 days. Control animals

15      received an equivalent volume (5 ml/kg/day) of the carboxymethylcellulose solution. At the end of the experiments, the animals were sacrificed under ether anesthesia. Adipose tissue was immediately removed and frozen in liquid nitrogen.

**RNA analysis**

20      RNA extraction and Northern blot analyses were carried out according to the previously described method (Staels, B. et al., Arteriosclerosis and Thrombosis 1992, 12(3): 286-294) using rat Rev-erb alpha, murine PPAR gamma and murine aP2, chicken beta-actin and human 36B4 cDNA probes.

25      **Transfection experiments**

The constructs comprising fragments of the Rev-erb alpha gene promoter cloned in the promoter-less plasmid pGL2 or in plasmid SV40pGL2 (Promega, Madison, WI, USA) have been previously described (Adelman, G. et al., Proc. Natl. Acad. Sci. USA 1996, 93(8): 3553-3558). Human HepG2 hepatoma cells were obtained from the European

30      Collection of cultured animal cells (Porton Down, Salisbury, UK) and 3T3-L1 cells were from the American Type Cell Culture (ATCC). Cells were grown in DMEM medium supplemented with 2 mM glutamine and 10 % (vol/vol) fetal calf serum (FCS) in a humidified 5 % CO<sub>2</sub> atmosphere at 37°C. All transfections were done in triplicate.

Luciferase activity was determined on total cell extracts by using a luciferase test system (Promega, Madison, WI, USA).

*In vitro* translation and gel shift assays.

5 The pSG5-mPPAR-gamma, pSG5-mRXR-alpha and pSG5-hRev-erb alpha plasmids were transcribed *in vitro* with T7 polymerase and translated with the rabbit reticulocyte lysate system (Promega, Madison, WI, USA). Gel shift experiments with *REV-ERB ALPHA*, PPAR GAMMA and/or RXR ALPHA proteins were carried out as previously described (Gervois, P. et al., Molecular Endocrinology 1999, 13(3): 400-409; Vu-Dac, N. et al., J. 10 Biol. Chem. 1994, 269(49): 31012-31018). For the competition experiments, increasing quantities of the indicated unlabeled probe were added immediately before adding the labeled oligonucleotide. The complexes were resolved in 5 % polyacrylamide gels using 0.25 x TBE buffer (90 mM borate, 2.5 mM EDTA, pH 8.3) at room temperature. Gels were dried and exposed overnight at -70°C on Rayon-X film (XOMAT-AR, Eastman 15 Kodak, Rochester, NY, USA).

Viral production and infection.

GP+E86 virus packaging cells (Markowitz, D. et al., J. Virol. 1988, 62(4): 1120-1124) were grown in DMEM medium (4.5 g/l glucose) supplemented with 10 % heat-20 inactivated calf serum (HyClone, Logan, UT, USA), 8 µg/ml gentamicin, 50 U/ml penicillin, 50 µg/ml streptomycin, at 37°C in a 95 % humidified air/5 % CO<sub>2</sub> atmosphere. To generate cell lines constitutively overexpressing the *REV-ERB ALPHA* receptor, the sequence coding for the *REV-ERB ALPHA* receptor was inserted upstream of the internal ribosome entry site and the neomycin resistance gene pCITE (Novagen, Madison, WI, 25 USA) of the retroviral plasmid MFG (Dranoff, G. et al., Proc. Natl. Acad. Sci. USA, 1993, 90(8): 3539-3543) using NcoI-BamHI sites, to produce the pMFG-Rev-erb alpha plasmid.

A similar construct in which the *REV-ERB ALPHA* receptor sequence was absent was used throughout the study as control (pMFG-Neo).

30 The bicistronic construct was designed to allow simultaneous expression of the *REV-ERB ALPHA* receptor and the neomycin resistance gene product in infected cells. To produce recombinant virus, GP+E86 cells (15,000/cm<sup>2</sup>) were transfected with the MFG plasmid constructs (2 µg) using lipofectamine (Life Technologies-Invitrogen, Groeningen, The

Netherlands) and selecting resistant clones with the geneticin analog G418 (0.8 mg/ml, Life Technologies-Invitrogen, Groeningen, The Netherlands).

3T3-L1 cells were infected with MFG-Neo or MFG-Rev-erb alpha virus produced by GP+E86 cells as described (Mattot, V. et al., Oncogene 2000, 19(6): 762-772) and 5 selected for resistance to geneticin until establishment of stable lines (approximately 10 days).

#### Cell culture and differentiation.

10 3T3-L1 cells (obtained from the ATCC) were cultured in DMEM growth medium supplemented with 10 % fetal calf serum. Cells were differentiated by the method of Bernlohr et al. (Bernlohr, D.A. et al., Proc. Natl. Acad. Sci. USA 1984, 81(17): 5468-5472).

15 Cells post-confluent after two days of culture (designated day D0) were transferred to differentiation medium (DMEM, 10 % FCS, 1  $\mu$ M dexamethasone, 10  $\mu$ g/ml insulin and 0.5 mM 3-methyl-1-isobutylxanthine (IBMX) (Sigma, St Louis, MO, USA)) for two days. Cells were then grown in post-differentiation medium (DMEM, 10 % FCS, insulin) with or without rosiglitazone. The medium was changed every day. Stable 3T3-L1 pre-adipocytes expressing the *REV-ERB ALPHA* receptor were grown in the same conditions but differentiated without dexamethasone. After the treatment, cells were fixed with 10 % 20 formaldehyde in PBS and stained with Oil Red O (Sigma, St Louis, MO, USA). Alternatively, total RNA was extracted as described earlier.

## RESULTS

### Activation of the PPAR GAMMA receptor increases expression of the *REV-ERB ALPHA* receptor in rat adipose tissue.

25 To determine whether activation of the PPAR GAMMA receptor has an effect on *REV-ERB ALPHA* receptor expression *in vivo*, rats were treated for 14 days with rosiglitazone (designated BRL), a highly specific, active ligand of the PPAR GAMMA receptor. *REV-ERB ALPHA* receptor expression was analyzed in epididymal and perirenal adipose tissue 30 by Northern blot. In comparison with the control, treatment with rosiglitazone sharply increased the levels of *REV-ERB ALPHA* receptor mRNA in the adipose tissues studied (Figure 1). Levels of beta-actin mRNA used as control were unaffected by the treatment.

These experiments show that activation of the PPAR GAMMA receptor by rosiglitazone increases *REV-ERB ALPHA* receptor expression in adipose tissue.

Activation of the PPAR GAMMA receptor induces *REV-ERB ALPHA* receptor mRNA in 5 3T3-L1 pre-adipocytes.

To investigate the molecular mechanism of this induction, the inventors studied the regulation of expression of *REV-ERB ALPHA* receptor mRNA by rosiglitazone in 3T3-L1 pre-adipocytes (Figure 2). 3T3-L1 pre-adipocytes were grown to confluence in a medium supplemented with 10 % fetal calf serum. Confluent cells were transferred to a medium containing lipid-depleted serum and cells were differentiated with a mixture containing dexamethasone, IBMX, insulin, with or without rosiglitazone (1  $\mu$ M).

10 Levels of *REV-ERB ALPHA* receptor mRNA increased as the pre-adipocytes differentiated to adipocytes. However, compared with the standard differentiation treatment, *REV-ERB ALPHA* receptor mRNA levels were induced earlier when 15 rosiglitazone was present. Said levels were significantly higher after 9 days in fully differentiated 3T3-L1 adipocytes.

Beta-actin mRNA levels used as control were practically unchanged during adipogenesis and were not affected by treatment with rosiglitazone.

20 The PPAR GAMMA receptor induces *REV-ERB ALPHA* receptor expression at the transcriptional level.

25 To clarify whether induction of *REV-ERB ALPHA* receptor mRNA takes place at the level of transcription, the inventors tested the effects of overexpression of the PPAR GAMMA receptor and stimulation by rosiglitazone on the transcriptional activity of a construct comprising a luciferase reporter gene under the control of a 1.7 kb fragment of the Rev-erb alpha gene promoter.

30 3T3-L1 cells were transfected with the construct comprising the luciferase reporter gene under the control of a 1.7 kb fragment of the Rev-erb alpha gene promoter in the presence of a pSG5-PPAR-gamma expression vector enabling expression of the murine PPAR GAMMA receptor, or the corresponding empty vector pSG5, and treated with rosiglitazone or the excipient.

The activity of the Rev-erb alpha gene promoter was induced by overexpression of the PPAR GAMMA receptor, an effect which moreover was amplified in the presence of rosiglitazone (Figure 3). On the other hand, when the construct comprising the luciferase reporter gene under the control of a 1.7 kb fragment of the Rev-erb alpha gene promoter 5 was transfected alone, no effect was observed. These findings indicate that transcription of the Rev-erb alpha gene is induced by rosiglitazone via activation of the PPAR gamma receptor.

An element named Rev-DR2, showing strong homology with a “DR2” response element of a nuclear receptor, was identified in the Rev-erb alpha gene promoter. It was shown 10 that said *REV-ERB ALPHA* receptor binds to this site and represses its own transcription via the site (Adelman, G. et al., Proc. Natl. Acad. Sci. USA 1996, 93(8): 3553-3558). This was also identified as the response element to which the PPAR alpha/RXR ALPHA heterodimer binds to confer a fibrate response to the Rev-erb alpha gene in liver (Gervois et al., Mol. Endocrinol. 1999, 13: 400-409).

15 To confirm that the Rev-DR2 site can also function as a response element of the PPAR GAMMA receptor in adipose tissue, the inventors conducted transient transfection experiments using constructs comprising wild-type and truncated versions of the Rev-erb alpha gene promoter named pGL2-hRev-erb $\alpha\delta$  and pGL2-hRev-erb $\alpha\Delta$  described previously (Adelman, G. et al., Proc. Natl. Acad. Sci. USA 1996, 93(8): 3553-3558) 20 (Figure 4). To confirm that the Rev-DR2 site can also function as a response element of the PPAR GAMMA receptor in adipose tissue, the inventors also carried out transient transfection experiments using the previously described constructs (Adelman, G. et al., Proc. Natl. Acad. Sci. USA 1996, 93(8): 3553-3558) comprising wild-type and mutated versions of the Rev-DR2 site cloned upstream of the SV40 promoter (Rev-DR2, Rev-25 DR2M5’ and Rev-DR2M3’) (Figure 4). Cotransfection of HepG2 cells with a PPAR GAMMA receptor expression vector and a reporter vector comprising two copies of the wild-type Rev-DR2 site cloned upstream of the SV40 promoter and a luciferase reporter gene led to a 2.5-fold greater induction of transcriptional activity as compared to the level observed with the empty pSG5 vector. On the other hand, no effect was seen when the 30 reporter vector comprised two copies of the mutated Rev-DR2 site in either 3’ or 5’ position. The effect of overexpression of the PPAR GAMMA receptor was amplified in the presence of rosiglitazone. These results clearly demonstrate that the activity of the

Rev-erb alpha gene promoter is regulated by the PPAR GAMMA receptor and that this induction is effected through the Rev-DR2 site (Figure 4).

5 The PPAR GAMMA receptor binds as a heterodimer with the RXR ALPHA receptor to the Rev-DR2 site.

Lastly, binding of the PPAR GAMMA receptor to the Rev-DR2 site was studied. A test to measure electrophoretic mobility (gel shift or electrophoretic mobility shift assay) was carried out using PPAR GAMMA and RXR ALPHA proteins synthesized *in vitro*. As control, the *in vitro*-produced *REV-ERB ALPHA* receptor was shown to bind to the wild-

10 type Rev-DR2 site as both monomer and homodimer (Figure 5). In contrast, no binding was observed when the Rev-DR2 oligonucleotide bore a mutation on the AGGTCA half-site located 5' (M5') as previously described (Adelman, G. et al., Proc. Natl. Acad. Sci. USA 1996, 93(8): 3553-3558). Finally, the *REV-ERB ALPHA* receptor bound as a monomer to the Rev-DR2 site bearing a mutation on the AGGTCA half-site located 3' 15 (M3') as previously described (Adelman, G. et al., Proc. Natl. Acad. Sci. USA 1996, 93(8): 3553-3558). The RXR ALPHA or PPAR GAMMA receptors alone did not bind to any of the oligonucleotides indicating that PPAR GAMMA and RXR ALPHA cannot bind in monomeric form. Binding to the Rev-DR2 site was seen when the PPAR GAMMA receptor was incubated with the RXR ALPHA receptor. Binding was specific 20 because it could be competed with an excess of unlabeled oligonucleotide. In contrast, the PPAR GAMMA/RXR ALPHA complex did not bind to the mutant Rev-DR2 site (M5' or M3'). These binding studies show that PPAR GAMMA binds as a heterodimer with RXR ALPHA to the intact Rev-DR2 site of the Rev-erb alpha gene promoter.

25 The *REV-ERB ALPHA* receptor increases the adipogenic activity of the PPAR GAMMA receptor.

To directly confirm the participation of the *REV-ERB ALPHA* receptor in adipogenesis, the entire cDNA encoding the *REV-ERB ALPHA* receptor was cloned in a retroviral vector. 3T3-L1 pre-adipocytes were then infected with the resulting virus. Stable cell 30 lines established by antibiotic selection with G418 (neomycin) after infection with either MFG-Neo virus (negative control) or MFG-Rev-erb alpha virus, were grown to confluence and then treated with a differentiation medium (designated Mix) containing IBMX, insulin with or without rosiglitazone (BRL) (1  $\mu$ M). Endogenous or viral-induced

expression of *REV-ERB ALPHA* was checked by immunocytochemical analysis or by Western blot (Figure 6E). Cells infected with MFG-Neo expressed high levels of *REV-ERB ALPHA* receptor in comparison with MFG-Neo-infected control cells.

In the absence of rosiglitazone, exogenous expression of the *REV-ERB ALPHA* receptor  
5 induced only a weak morphological differentiation of the pre-adipocytes. In the presence  
of rosiglitazone (1  $\mu$ M), there was an increase in pre-adipocyte differentiation and in lipid  
accumulation in cells expressing the *REV-ERB ALPHA* receptor as compared with control  
cells. In fact, after fixation and staining with Oil red O, a weak accumulation of lipids  
was observed in the absence of rosiglitazone, but high lipid accumulation was seen in  
10 cells expressing the *REV-ERB ALPHA* receptor treated with rosiglitazone for 8 days  
(Figures 6A - 6D). To obtain the same result with rosiglitazone alone without hormonal  
stimulation, the cells had to be differentiated for 16 days (data not shown).

These morphological changes occurred in parallel to a similar variation in mRNA levels  
15 of adipocyte-specific markers. Northern blot analyses showed a weak but significant  
expression of the PPAR GAMMA receptor and aP2 in cells expressing the *REV-ERB*  
*ALPHA* receptor (Figure 7).

In a surprising manner, endogenous levels of the *REV-ERB ALPHA* receptor were  
perturbed in cells overexpressing the *REV-ERB ALPHA* receptor. aP2 and PPAR  
20 GAMMA receptor mRNA levels were high in cells expressing the *REV-ERB ALPHA*  
receptor treated with a rosiglitazone mixture after only four days of differentiation (Figure  
7), or with rosiglitazone alone (Figure 7). This phenomenon was not observed until 8-12  
days in control cells. These findings show that exogenous expression of the *REV-ERB ALPHA*  
receptor has a weak effect without hormonal stimulation and amplifies induction  
of adipogenesis by PPAR GAMMA ligand activation.

25

It is in this manner that the use of the pre-adipocyte cell line of the invention,  
overexpressing the *REV-ERB ALPHA* receptor, enabled identification of the Rev-erb  
alpha gene as a novel target gene for the PPAR GAMMA receptor in the adipogenic  
transcription factor cascade. This observation of the active role of the *REV-ERB ALPHA*  
30 receptor in the adipocyte differentiation process allowed the inventors to develop a novel  
screening method to identify active compounds involved in adipocyte modulation.